Journal of Experimental Microbiology and Immunology (JEMI) Vol. 22:1-15

Exposure of Exponential Phase *Escherichia coli* to Ampicillin and Gentamicin Does Not Confer Cross-Protection Against T7 Bacteriophage-Induced Lysis

Amanda Krystal, Reiko Okamoto, Andrea Sze, Zachary Weiss

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY Bacteria like *Escherichia coli* employ sensing and gene regulatory systems to respond to stress. The sigma factor RpoS has been shown to play a role in response to stressors like antibiotics. Previous investigations have shown that exposure to a stressor may lead to increased tolerance to a subsequent stressor. This phenomenon, termed cross-protection, has been demonstrated in *E. coli*, whereby treatment with sub-lethal concentrations of antibiotics delayed T7 bacteriophage-induced lysis. Here we re-examine this cross-protection model and, given its role in the general stress response in *E. coli*, hypothesize RpoS to be a central mediator of this phenomenon. Using a modified phage lysis assay, we show that delayed T7 phage-induced lysis may not occur in exponential phase cells pretreated with sub-lethal concentrations of ampicillin and gentamicin. In addition, we show that the absence of RpoS does not result in greater susceptibility to T7 phage-induced lysis, suggesting that RpoS may not play a meaningful role in the mediation of cross-protection in exponential phase cells. Along with our results, we propose a complex model of cross-protection, whereby sub-lethal levels of antibiotics, growth phase, stress, and released soluble factors like outer membrane vesicles all play an as-of-yet unclear role in a larger system of bacterial response to stress.

INTRODUCTION

To respond to both internal and external cell stress, bacteria like *Escherichia coli* utilize a diverse array of tools to alter gene expression. RpoS (σ^S) is an *E. coli* sigma factor which is upregulated during periods of stress (1, 2). Sigma factors bind to RNA polymerase (RNAP), enabling RNAP to associate with particular promoter regions and initiate transcription depending on which sigma factor is bound. This system allows the cell to respond to varying conditions through the transcription of different sets of genes (2).

Recent studies have shown that prior exposure to a stressor may lead to increased tolerance to subsequent stressors through a mechanism referred to as cross-protection (3). Because of this, there has been interest in the mechanisms by which cross-protection may be induced (6, 7). Given the central position of RpoS in mediating response to stress, there exists the potential for it to be involved in cross-protection. The role of RpoS in promoting cross-protection is not well understood.

Bacteriophages (phages) are viruses that specifically infect bacteria and archaea, and are heavily used in molecular biology (4). Many phages initiate what is known as a lytic cycle (4). In this cycle, the phage injects its genome into a bacterial host where expression of viral components needed for its replication can occur. Following packaging of viral progeny, the final stage of infection is the rupture of the host cell via viral egress, resulting in cell death. The bacteriophage species T7 belongs to the Podoviridae family of viruses and is among the most studied of phage species (4). T7 phage undergoes a lytic cycle that is known to infect *E. coli*. The mechanisms by which bacteria like *E. coli* respond to T7 phage-induced lysis is dependent on a wide-range of factors, including host cell metabolism, growth phase, and external environmental conditions (5).

A previous study demonstrated cross-protection through the treatment of *E. coli* B23 cells with sub-lethal concentrations of aminoglycoside and beta-lactam antibiotics followed by subsequent exposure to T7 phage (6). Cells treated with antibiotics prior to T7 phage exposure exhibited delayed lysis compared to cells that were not exposed to the antibiotics (6). Furthermore, another recent study provided evidence for a link between aminoglycoside

Published Online: 24 August 2018

Citation: Krystal A, Okamoto R, Sze A, Weiss Z. 2018. Exposure of Exponential Phase *Escherichia coli* to Ampicillin and Gentamicin Does Not Confer Cross-Protection Against T7 Bacteriophage-Induced Lysis. JEMI 22:1-15

Editor: Julia Huggins, University of British Columbia

Address correspondence to: https://jemi.microbiology.ubc.ca/

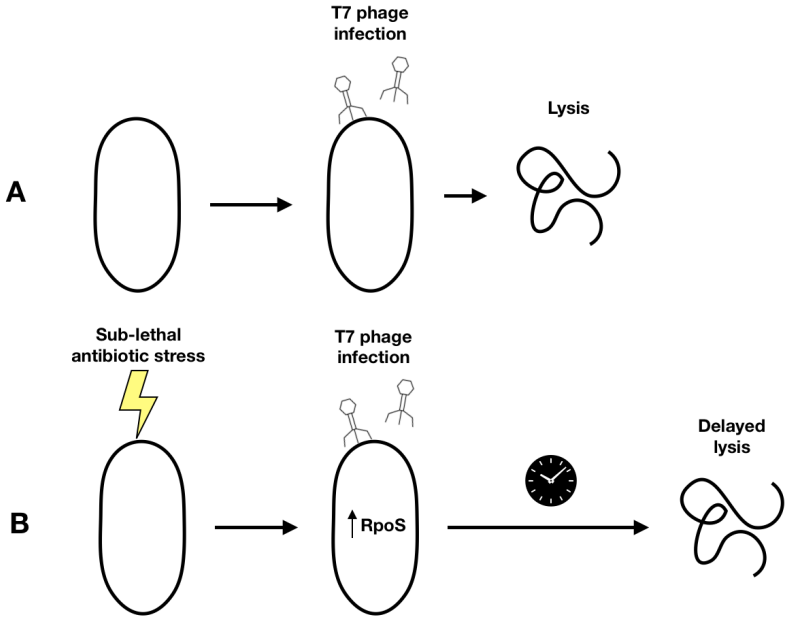

FIG. 1 Proposed model of RpoS-mediated cross-protection. (A) In the absence of any prior stressor, RpoS is not induced at the onset of T7 phage infection, leading to expected cell lysis. (B) Sub-lethal antibiotic stress induces upregulation of RpoS. RpoS confers cross-protection to T7 phage infection, leading to delayed lysis compared to untreated cells.

exposure to *E. coli* and the release of protective factors, known as outer membrane vesicles (OMVs), via a stress-induced signal transduction pathway (7). In the case of subsequent exposure to T7 phage, it has been hypothesized that these factors may prevent infection (7).

Here we examine the role of RpoS as a potential mediator in cross-protection against T7 phage-induced lysis following exposure to sub-lethal concentrations of antibiotics. We proposed that, in contrast to untreated cells, cells treated with sub-lethal concentrations of antibiotics would exhibit delayed T7 phage-induced lysis due to cross-protection conferred through the upregulation of RpoS (Figure 1). Through a gradient antibiotic concentration phage lysis assay, we find that the delayed T7 phage-induced lysis does not occur in exponential phase cells pretreated with sub-lethal concentrations of ampicillin or gentamicin, contradictory to previous results. We also show that loss of RpoS does not result in greater susceptibility to T7 phage-induced lysis. Ultimately, our results may point toward a more intricate model of cross-protection, and provide valuable insight for groups hoping to further elucidate the role of RpoS in response to antibiotics and the mechanism of cross-protection.

METHODS AND MATERIALS

Bacterial strains and bacteriophage used in this study. *E. coli* K-12 wild type (BW25113) and *rpoS* knockout mutant (JW5437) strains were obtained from the in-house collection at the Department of Microbiology at the University of British Columbia. The single-gene knockout mutant contains a kanamycin resistance (Kan^R) cassette in place of the *rpoS* gene,

Table 1 Forward (F) and reverse (R) primers for target genes

Target gene	Primer sequences (5'- 3')
T7 *gp10a*	F: CGA GGG CTT AGG TAC TGC
	R: GGT GAG GTG CGG AAC TTC
T4 *gp23*	F: GCC ATT ACT GGA AGG TGA AGG
	R: TTG GGT GGA ATG CTT CTT TAG
pUC19 *bla*	F: GTG AAA TAC CGC ACA GAT GC
	R: GGC GTT ACC CAA CTT AAT CG

which is flanked by FLP recognition target sites. In addition, *E. coli* BT340, carrying the pCP20 plasmid, was obtained from the in-house collection. For the purposes of this paper, strains BW25113 and JW5437 are referred to as wild type (WT) and Δ*rpoS*, respectively. Δ*rpoS* following Kan^R cassette removal is referred to as $\Delta rpoS^{KAN}$. All strains were grown overnight in LB at 37ºC on a shaker (200 rpm) prior to use. T7 phage stock used for subsequent propagation was provided by Fettig *et al.* (8).

Antibiotic stock preparation. Stock solutions of four antibiotics were made. Ampicillin, penicillin, and gentamicin stock solutions were prepared at a concentration of 50 mg/mL in dH_2O, while the stock solution of kanamycin was prepared at a concentration of 10 mg/mL in dH_2O. Antibiotic solutions were filter sterilized with a 0.22 µm pore size and stored at -20ºC. All working solutions were made using dilutions of these stock solutions.

WT, Δ*rpoS*, and $\Delta rpoS^{KAN}$ growth curves. A subculture was prepared by adding 500 µL of overnight culture to 50 mL of LB. The culture was incubated at 37ºC on a shaker (200 rpm). Optical density readings at 600 nm (OD_{600}) were then taken every 30 minutes using the Ultrospec 3000 until three consecutive readings showed minimal change. At this time, the cells were said to have reached stationary phase. A conversion factor of 1 OD_{600} = 8 x 10^8 CFU/mL was used to determine the cell concentration at each time point.

T7 phage propagation. T7 phage was propagated by adapting the procedures used by Pelzek *et al.* (4). 50 µL of WT overnight culture was added to 5 mL of pre-warmed LB and then incubated at 37ºC on a shaker (200 rpm) until an OD_{600} of 0.5 was measured. 1 mL of T7 phage from Fettig *et al*. was added to the culture prior to overnight incubation (8). The following day, 1% (w/v) chloroform was added to the overnight culture to induce cell lysis and phage release. The solution was centrifuged for 10 minutes at 2750 x g at 4ºC, and the supernatant was filter sterilized with a 0.22 µm pore size. The phage was stored at 4ºC.

Double agar overlay plaque assay to determine T7 phage titre. The T7 phage titer was quantified according to the procedures used by Francis *et al.* (9). 50 µL of WT overnight culture was added to 5 mL of pre-warmed LB. The subculture was incubated at 37ºC on a shaker (200 rpm) until mid-exponential phase (OD_{600} ~ 0.5) was reached. 100 µL of the culture was then mixed with 100 µL of T7 phage of varying dilutions (undiluted, 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}). Three replicates of each dilution were incubated for 10 minutes. Each mixture was added to 3 mL of the overlay 0.75% (w/v) LB agar, vortexed briefly, and then poured onto the underlay 1.5% (w/v) LB agar. Plates were incubated at 37ºC overnight and the plaques were quantified the following day.

PCR and agarose gel electrophoresis to confirm T7 phage stock purity. PCR was set up according to the Thermo Fisher Platinum *Taq* DNA Polymerase manual (10). PCR master mix was made up of 35 µL of 10X buffer, 10.5 µL of 50 mM $MgCl_2$, and 7 µL of 10 mM dNTP mix. The final reaction volume was 50 µL and each experimental reaction was comprised of 7.5 µL of the master mix, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 0.2 µL of *Taq* polymerase, 2 µL of T7 phage, and 38.3 µL of PCR-grade H_2O. Two sets of primers were used in the experimental reactions: one targeting T7 phage *gp10a* to confirm the presence of T7 phage and another targeting T4 phage *gp23* to detect any possible contamination with T4 phage (Table 1). Primers specific to T7 *gp10a* and T4 *gp23* were expected to yield PCR products 296 bp and 398 bp in size respectively (11). Various amounts of pUC19 plasmid with primers specific to *bla* were used as positive controls.

PCR was performed in the Bio-Rad T100 Thermal Cycler. Nucleic acids were amplified using the following program: initial denaturation at 95ºC for 5 minutes; denaturation at 95ºC for 30 seconds; annealing at 50ºC for 30 seconds; and extension at 68ºC for 60 seconds. Denaturation, annealing, and extension steps were repeated for 30 cycles, followed by the final extension step at 68ºC for 5 minutes. Amplicons were stored at 12ºC overnight.

Amplicons were run on a 2% agarose gel to visualize the presence of T4 phage contamination. 0.5 g of agarose was mixed with 25 mL of Invitrogen SYBR Safe DNA Gel Stain in 0.5X TBE. 2 µL of the 10X gel loading buffer was added to 18 µL of each PCR

product. 12 µL of each sample and 10 µL of the Invitrogen 100 bp DNA Ladder were loaded onto the gel. The gel was run at 70 V for 20 minutes and then the voltage was increased to 80 V. The gel was then run for 40 minutes.

Isolation and quantification of the pCP20 plasmid. 5 mL of LB supplemented with 10 µL of ampicillin (50 mg/mL) was inoculated with *E. coli* BT340 and incubated overnight at 30°C on a shaker (200 rpm). The pCP20 plasmid was isolated from the overnight culture using the Bio Basic EZ-10 Spin Column Plasmid DNA Miniprep Kit following the attached manual (12). Plasmid concentration and purity were assessed using the Thermo Scientific NanoDrop 2000 and stored at -20°C.

Excision of the Kan^R cassette from Δ*rpoS* using Flp recombinase. Electrocompetent cells were prepared according to the procedure of the Barrick Lab (13). Multiple transformation reactions were performed by adapting the procedure used by the Barrick Lab (14). 10, 50, 100, and 360 ng of the pCP20 plasmid carrying the yeast Flp recombinase gene were transformed into 50 µL aliquots of electrocompetent Δ*rpoS* cells. Competent cells were transformed with 10 and 100 ng of the pUC19 plasmid to serve as positive controls. As for the negative control, the competent cells were transformed with dH_2O. After electroporation with the Bio-Rad MicroPulser Electroporator set to program Ec2, the cells were recovered with the addition of 500 µL of LB and 1 µL of ampicillin (50 mg/mL). Cells were incubated at 30ºC on a shaker (200 rpm) for 1 hour. Following this treatment, 20 µL of each sample was spread on LB and LB-Amp (50 µg/mL) plates and then incubated at 30ºC overnight.

Induction of Flp recombinase was performed by adapting the procedure used by the Barrick Lab (15). To begin this process, a colony from the LB-Amp plate containing cells electroporated with 100 ng of the pCP20 plasmid was streaked onto a new LB-Amp plate and incubated at 30ºC overnight. A colony from the restreaked plate was used to inoculate 5 mL of LB, which was grown overnight at 43ºC to induce Flp recombinase expression and subsequent plasmid loss. 10^{-4}, 10^{-6}, and 10^{-8} dilutions of the overnight culture were made and 50 µL of each dilution was plated in duplicate on LB and incubated overnight at 30ºC. In order to screen for DNA recombination and plasmid loss, LB-Kan (50 µg/mL), LB-Amp (50 µg/mL), and LB plates were each divided into six sections. Six colonies from the plate containing the 10^{-6} dilution of the overnight culture were selected and each colony was patched in the following order: LB-Kan, LB-Amp, and LB. Following patch plating, the LB-Kan and LB plates were incubated overnight at 37ºC while the LB-Amp plate was incubated overnight at 30ºC.

Minimum inhibitory concentration assay. 10^{-2} dilution of the WT overnight culture was incubated to mid-exponential phase (OD_{600} ~ 0.6) at 37ºC on a shaker (200 rpm). 100 µL of LB was dispensed into each well in a clear, polystyrene 96-well plate, with the exception of the last row, which was left empty. Serial dilutions of antibiotics were prepared by dispensing 100 µL of ampicillin (256 µg/mL), gentamicin (256 µg/mL), kanamycin (256 µg/mL), and penicillin (256 µg/mL and 3072 µg/mL) into the first well of the appropriate row. The contents of each well were thoroughly mixed, followed by the transfer of 100 µL into the adjacent well of that row. This process was repeated until the last well of the row, where the remaining 100 µL was discarded after mixing. For each row of ampicillin, kanamycin, and gentamicin, the concentrations ranged from 0.0625 µL/mL to 128 µg/mL. For penicillin, the concentrations ranged from 0.0625 µg/mL to 1536 µg/mL across two rows. The first and second rows were not loaded with antibiotics to serve as controls. WT culture was added to all wells containing LB, except for the first row. Since the assay was performed in duplicate, 5 µL of WT culture was dispensed into the wells of one plate while 10 µL was used for the other. The plate was incubated at 37°C for 24 hours and then read with the Epoch Microplate Spectrophotometer at 600 nm. The lowest antibiotic concentration that inhibits bacterial growth (i.e. MIC) was determined to be the antibiotic concentration that resulted in an OD_{600} reading that was equal to or less than the negative control reading. The sub-lethal antibiotic concentration was calculated to be half of the MIC in accordance with the definition used by Li *et al.* (6).

Phage lysis assays. Two models were used. In the first model, the assay described by Li *et al*. was adapted (6). WT *E. coli* were inoculated into LB supplemented with sub-lethal concentrations of ampicillin (8 µg/mL), penicillin (144 µg/mL), kanamycin (16 µg/mL), or gentamicin (48 µg/mL) and incubated overnight at 37ºC on a shaker (200 rpm). Overnight cultures that exhibited growth were diluted 10^{-1} and supplemented with the appropriate antibiotic and incubated to mid-exponential phase at 37ºC on a shaker (200 rpm). Outgrowths were normalized to an OD_{600} of 0.24 (the lowest OD_{600} value of the samples after 3 hours of growth) with LB. To prepare T7 phage, 2 µL of phage was diluted in 998 µL dH_2O and 4 µL of phage was diluted in 996 µL of dH_2O to obtain multiplicities of infection (MOI) of 0.05 and 0.1, respectively. A 96-well plate was loaded as follows (Figure S1). 100 µL of LB alone was used as the blank. 100 µL of WT outgrowth was used as a control for expected growth without antibiotics or T7 phage. 100 µL of WT cells treated with sub-lethal concentrations of ampicillin or penicillin were used as a control for expected growth with antibiotics. 90 µL of WT outgrowth grown without antibiotics with 10 µL of T7 phage was used as a control for expected lysis. For the final rows, 10 µL of T7 phage was added to 90 µL of WT outgrowth grown in the presence of either the sub-lethal concentration of ampicillin or penicillin. The first six columns were treated with an MOI of 0.1 and the last six columns were treated with an MOI of 0.05. Controls were replicated over six wells, while treatments were replicated over 12 wells. The plate was measured on the Epoch Microplate Spectrophotometer at 600 nm every 10 minutes over the course of 120 minutes and incubated at 37°C between reads.

In the second model, the gradient assay, WT *E. coli* were incubated in LB alone or LB with varying concentrations of ampicillin (0.5, 1, 2, 4, and 8 µg/mL) or gentamicin (0.25, 0.5, 1, 2, 4, and 8 µg/mL). Overnight cultures were diluted 10^{-1} with the appropriate antibiotic and grown to mid-exponential phase at 37ºC on a shaker (200 rpm). Outgrowths were normalized to an OD_{600} ~ 0.3 with LB. T7 phage (MOI of 0.1) was prepared as described in the previous section. A clear, polystyrene 96-well plate was loaded as follows (Figure S2). 100 µL of *E. coli* outgrowth was used as a control for expected growth without antibiotics or phage. 90 µL of *E. coli* outgrowth grown in the absence of antibiotics with 10 µL of T7 phage was used as a control for expected lysis. For the subsequent rows, 90-100 µL of outgrowth supplemented with the specified antibiotic concentration were added. Outgrowth supplemented with ampicillin was added to the first six wells of each row (100 µL for first three wells, 90 µL for the last three) and outgrowth supplemented with gentamicin was added to the remaining six wells of each row (100 µL for first three wells, 90 µL for the last three). 10 µL of T7 phage was then added to all wells previously filled with 90 µL of outgrowth. Controls were replicated over 12 wells, treatments without T7 phage were replicated over three wells, and treatments with T7 phage were replicated over three wells. The plate was measured on an Epoch Microplate Spectrophotometer at 600 nm every 10 minutes over the course of 110-120 minutes and incubated at 37°C between reads. To test $\Delta rpoS^{KAN}$, gentamicin was used at concentrations of 0.125, 0.25, 0.5, 1, 2, 4, and 8 µg/mL. Incubation of the outgrowth culture was extended from 2 hours to 4 hours to account for the slower growth of this strain.

Western blot analysis. WT and $\Delta rpoS^{KAN}$ were inoculated into 1 mL of LB and incubated overnight at 37°C on a shaker (200 rpm). 10^{-1} dilution of the overnight culture was incubated for 3 hours at 37ºC on a shaker (200 rpm). Cells were pelleted using an Eppendorf Microcentrifuge and resuspended in 40 µL of 2X Laemmli Sample Buffer (1:19, 2-mercaptoethanol to 2X Laemmli Sample Buffer). The solution was then boiled on a heating block for 5 minutes. 25 µL of each sample and 10 µL of the All Blue Prestained and Unstained Protein Standards were loaded onto Bio-Rad Mini-PROTEAN TGX Stain-Free Precast Gels. Upper and lower chambers were filled with Bio-Rad 1X Tris/Glycine/SDS buffer. The gel was run at 300 V for 15 minutes and then activated using the Bio-Rad ChemiDoc MP Imaging System. The gel was transferred onto the Bio-Rad Trans-Blot Turbo Mini PVDF Transfer Pack following the protocol provided by the manufacturer, and then placed in the Bio-Rad Trans-Blot Turbo Transfer System (16). The blot transfer was performed at 2.5 A and 25 V for 7 minutes. The PVDF membrane was rinsed with methanol and dH_2O and then visualized on the Bio-Rad ChemiDoc MP Imaging System to measure total protein. The membrane was placed in 1X TBST supplemented with 5% dried skim milk and blocked overnight at 4ºC. The membrane was washed twice in TBST for 7 minutes with agitation at room temperature.

Table 2 Antibiotic concentrations

	5 µL *E. coli*		10 µL *E. coli*		
	MIC (µg/mL)	Sub-Lethal (µg/mL)	MIC (µg/mL)	Sub-Lethal (µg/mL)	Used Sub-Lethal (µg/mL)
Ampicillin	16	8	16	8	8
Penicillin	384	192	192	96	144
Gentamicin	128	64	64	32	48
Kanamycin	32	16	32	16	16

All subsequent incubation and wash steps were performed at room temperature with agitation. The primary antibody, anti-*E. coli* RNAP Sigma S (BioLegend, Catalog No. 663704, Lot No. B248680), was reconstituted in 100 µL of dH_2O. The membrane was incubated in 15 µL of the primary antibody diluted in 15 mL of 1X TBST supplemented with 1% dried skim milk for 1 hour. The membrane was then washed four times in 1X TBST for 7 minutes. Following the wash step, the membrane was incubated in 1.5 µL of the secondary anti-mouse antibody diluted in 15 mL of 1X TBST for 1 hour. The membrane was then washed four times in 1X TBST for 7 minutes and then once in 1X TBS for 10 minutes. The membrane was then incubated in 3 mL of Detection Reagent 1 and 3 mL of Detection Reagent 2 for 1 minute. Following this, RpoS was detected on the Bio-Rad ChemiDoc MP Imaging System.

RESULTS

WT, Δ*rpoS*, Δ*rpoS*^{KAN} growth curves. Growth curves for WT and Δ*rpoS* were produced to determine the incubation time and OD_{600} value corresponding to exponential phase. These were determined to be ~3 hours and OD_{600} ~0.5 for WT, and ~4 hours and OD_{600} ~0.25 for Δ*rpoS* (Figure S3). Since the effect of removing the Kan^R cassette on growth rate was unclear, a partial growth curve of Δ*rpoS*^{KAN} was generated. Following Kan^R cassette removal, the time required to reach mid-exponential phase was ~4 hours, nearly identical to Δ*rpoS* (Figure S4).

Confirmation of T7 bacteriophage stock purity. Through the plaque assay, the titre of our T7 phage stock was determined to be 5.6 x 10^9 PFU/mL. In order to determine whether or not this T7 phage stock was contaminated with T4 phage, another commonly used phage in our laboratory, PCR and agarose gel electrophoresis were performed. Primers specific to T7 *gp10a* and T4 *gp23* yielding PCR products of 296 bp and 398 bp respectively were used. Following PCR, the amplicons were run on a 2% agarose gel and visualized (Figure 2). A ~300 bp band in lane 2 was observed in accordance with the expected amplicon size of 295 bp, confirming the presence of T7 phage. The absence of bands in lane 3 demonstrated that our T7 phage stock was not contaminated with T4 phage. The presence of bands at ~150 bp in lanes 4-6 (positive controls) was also observed, suggesting that the PCR performed optimally. These results confirmed the purity of our T7 phage stock for later experiments.

Sub-lethal antibiotic concentrations determined by the MIC assay result in complete cell death. In order to determine the appropriate sub-lethal concentrations of ampicillin, penicillin, gentamicin, and kanamycin for later phage lysis assays, a MIC assay was performed. Using a 96-well plate, the MIC was defined as the antibiotic concentration that resulted in an OD_{600} value that was equal to or less than the negative control ($OD_{600} \leq 0.04$) and the sub-lethal antibiotic concentration was defined as half the MIC (Figure S5). The sub-lethal concentrations of ampicillin and kanamycin determined by the assay were consistent in both trials (Table 2). The sub-lethal concentration of ampicillin was 8 µg/mL, 16 times higher than the value reported by Li *et al.* (6). The sub-lethal concentration of kanamycin was 16 µg/mL, 8 times higher than the previously reported value (6). In contrast, the sub-lethal concentrations of gentamicin and penicillin suggested by the assay varied across the trials.

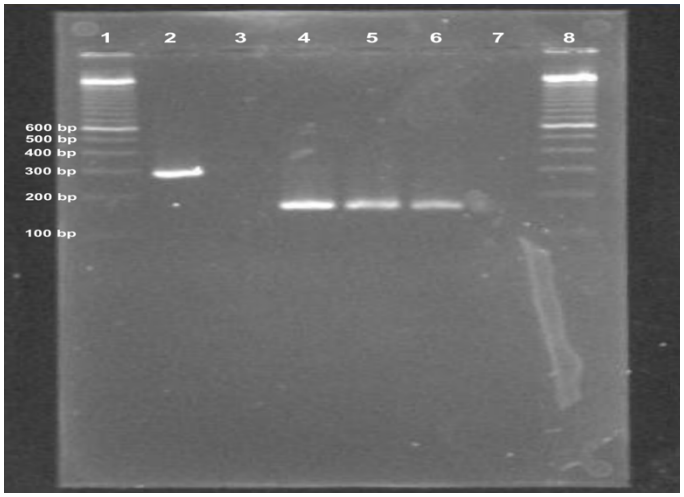

FIG. 2 Agarose gel (2%) electrophoresis confirmed purity of the T7 phage stock. Lane 1: Invitrogen 100 bp DNA Ladder; Lane 2: T7 + T7 *gp10a* primers; Lane 3: T7 + T4 *gp23* primers; Lane 4 (positive control): pUC19 (10 ng) + *bla* primers; Lane 5 (positive control): pUC19 (1 ng) + *bla* primers; Lane 6 (positive control): commercial pUC19 (10 ng) + *bla* primers; Lane 7: negative control; Lane 8: Invitrogen 100 bp DNA Ladder.

The suggested sub-lethal concentration of gentamicin varied from 32 µg/mL to 64 µg/mL, up to 64 times higher than the value reported Li *et al.* (6). In addition, the suggested sub-lethal concentration of penicillin ranged between 96 µg/mL and 192 µg/mL, up to 48 times higher than the previously reported value (6). These results, though confounding, were nonetheless used for subsequent experiments.

After determining the sub-lethal concentrations for each of the four antibiotics, we sought to reproduce the evidence supporting the occurrence of delayed lysis (6). WT cells were treated with sub-lethal concentrations of antibiotics and grown overnight. The following day, WT cells treated with kanamycin or gentamicin showed no growth, suggesting these sub-lethal concentrations determined from our MIC assay were too high. Thus, only cells treated with ampicillin or penicillin were used in the subsequent phage lysis assay. Ampicillin and penicillin-treated cells were subjected to T7 phage at MOIs of 0.1 and 0.05 in a 96-well plate and OD_{600} was measured over time (Figure S6). As expected, control wells not treated with T7 phage were observed to grow exponentially throughout the assay, while the OD_{600} of control wells treated with T7 phage began decreasing around t = 60 minutes. WT cells treated with one of ampicillin and penicillin, however, were not observed to grow over the duration of the assay, once again suggesting that the sub-lethal concentrations suggested by the MIC assay were too high. MOIs of 0.05 and 0.1 did not change the time at which a decrease in OD_{600} was observed.

Given these data, we suspected our calculated sub-lethal concentrations of all four antibiotics to be too high. This may have been a result of using a 96-well plate made out of a material that has a high binding affinity for antibiotics. In this scenario, the antibiotics may have been adsorbed onto the surface of the wells, consequently making it seem like the bacteria were able to withstand higher concentrations of antibiotics. As a result, we set out to develop a new assay in order to systematically assess the optimal concentrations of antibiotics to use in the assay to investigate the proposed phenomenon of delayed lysis.

T7 phage-induced lysis was not delayed in exponential phase WT following exposure to ampicillin. To find an optimal antibiotic concentration that could delay T7-phage induced lysis, a modified 96-well plate phage lysis was developed. In this gradient assay, WT cells were treated with varying concentrations of antibiotics and later infected with T7 phage (MOI of 0.1). With regard to WT cells treated with antibiotics in the absence of T7 phage, growth was almost entirely inhibited at the highest concentrations of ampicillin and gentamicin (Figures 3A, 3C). WT cells treated with 0.5, 1, and 2 µg/mL ampicillin exhibited growth comparable to that of WT cells treated with neither ampicillin nor T7 phage (Figure 3A). With respect to WT cells treated with only gentamicin, cell growth was inhibited as antibiotic concentration increased (Figure 3C). With regard to WT cells treated with both T7 phage and antibiotics, we observe peaks in OD_{600} which reflect the point at which the rate of T7 phage-induced lysis exceeds the rate of cell growth (Figures 3B, 3D). The times at which lysis began (i.e., time at which OD_{600} is highest) for the WT cells incubated with both T7 phage and

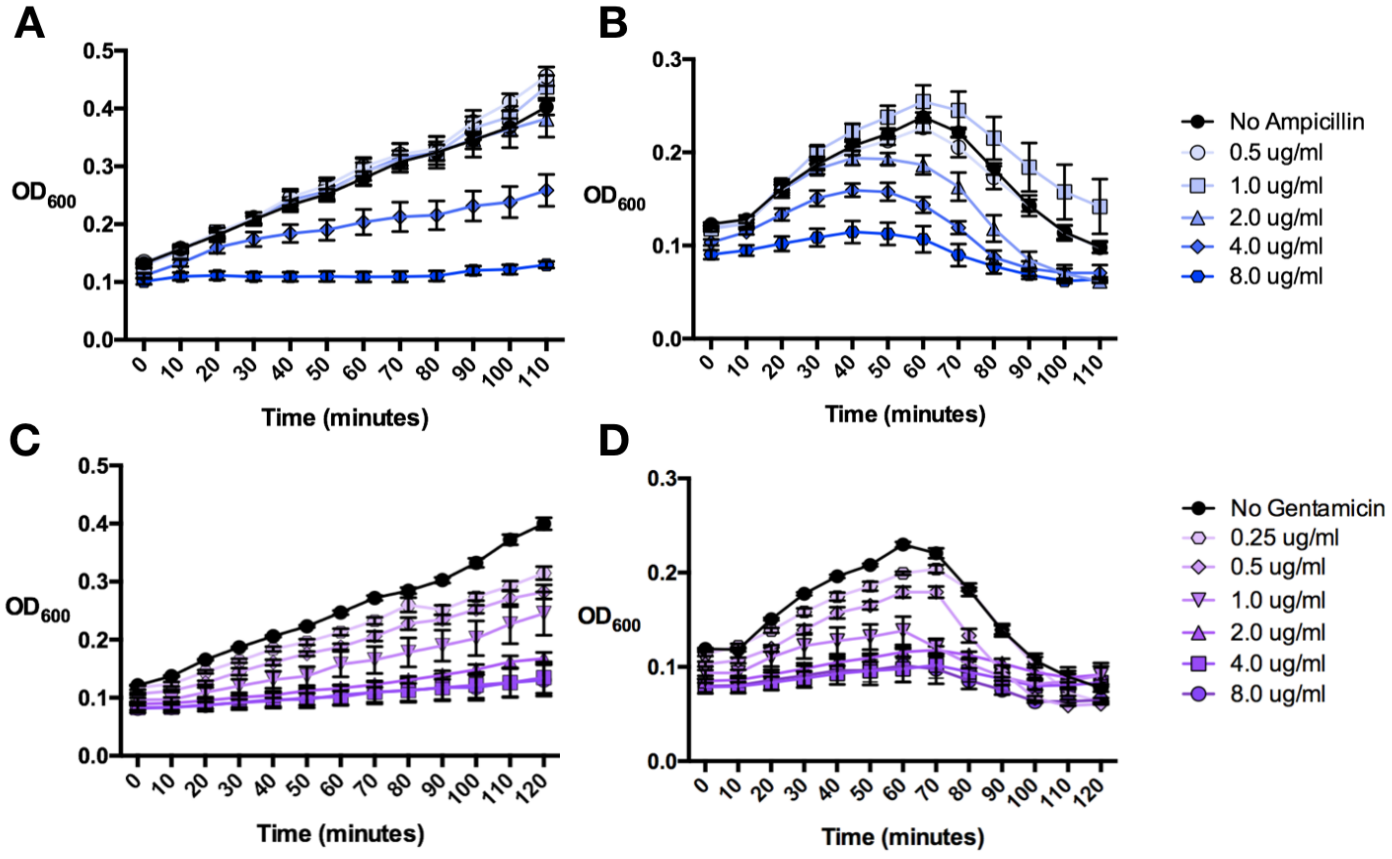

FIG. 3 Treatment with a gradient of concentrations of ampicillin and gentamicin does not induce delayed lysis in WT *E. coli* treated with T7 phage. (A) 96-well plate infectivity assay with WT *E. coli* treated with a gradient of concentrations of ampicillin in the absence of added phage. Bars represent +/- SEM. n = 36 wells for No Ampicillin condition. n = 12 wells for all ampicillin-treated conditions. (B) Gradient of concentrations of ampicillin in the presence of added phage at an MOI of 0.1. Bars represent +/- SEM. n = 42 wells for No Ampicillin condition. n = 12 wells for all ampicillin-treated conditions. (C) Gradient of concentrations of gentamicin in the absence of added phage. Bars represent +/- SEM. n = 36 wells for No Gentamicin condition. n = 3 wells for 0.25 μg/mL, n = 6 wells for 0.5 μg/mL and 8 μg/mL, and n = 9 for 1 μg/mL, 2 μg/mL, and 4 μg/mL. (D) Gradient of concentrations of gentamicin in the presence of added phage at an MOI of 0.1. Bars represent +/- SEM. n = 36 wells for No Gentamicin condition. n = 3 wells for 0.25 μg/mL, n = 6 wells for 0.5 μg/mL and 8 μg/mL, and n = 9 for 1 μg/mL, 2 μg/mL, and 4 μg/mL

ampicillin were either equal to, or earlier than, that of WT cells incubated with only T7 phage (Figure 3B). Likewise, the time at which lysis began for phage-treated WT cells exposed to 1 µg/mL gentamicin were similar to that of WT cells incubated with only T7 phage. However, for phage-treated WT cells incubated with 0.25, 0.5, 2, 4, or 8 µg/mL gentamicin, lysis was observed to be delayed by 10 minutes (Figure 3D). Taken together, these results suggest that exposure to ampicillin, regardless of concentration, does not delay T7 phage-induced lysis in exponential phase-WT cells. However, we remain uncertain about the delayed lysis observed in cells treated with gentamicin, as this was not consistently observed in all trials.

Absence of RpoS expression in *E. coli* growing in exponential phase may not affect T7 phage-induced lysis following exposure to ampicillin. To investigate the potential role of RpoS in cross-protection and delayed lysis, the gradient assay was repeated for Δ*rpoS*^{KAN} (i.e. single-gene knockout with the Kan^{R} cassette removed). With regard to Δ*rpoS*^{KAN} cells exposed to only antibiotics, cell growth was generally inhibited as antibiotic concentration increased (Figures 4A, 4C). With respect to Δ*rpoS*^{KAN} cells treated with both T7 phage and antibiotics, we observe peaks in OD_{600} which reflect the point at which the rate of T7 phage-induced lysis exceeds the rate of cell growth (Figures 4B, 4D). Similar to the observation in the previous gradient assay, the times at which lysis began for Δ*rpoS*^{KAN} cells treated with both T7 phage and ampicillin were either equal to, or earlier than, that of Δ*rpoS*^{KAN} cells

treated with only phage (Figure 4B). A different trend was observed for phage-treated Δ*rpoS*^{KAN} exposed to gentamicin (Figure 4D). With respect to the phage-treated Δ*rpoS*^{KAN} cells exposed to 0.25, 0.5, 4, and 8 µg/mL gentamicin, the times at which lysis began were similar to that of Δ*rpoS*^{KAN} cells only treated with phage. However, at 0.125, 1, 2 µg/mL gentamicin, the plots do not show distinct apexes. We suspect this result to be due to the slower growth observed in Δ*rpoS*^{KAN} compared to WT (Figures S3, S4). These results suggest that the absence of RpoS in *E. coli* during exponential phase may not affect viral lysis following ampicillin treatment. However, the effect of the absence of RpoS on phage-induced lysis following gentamicin treatment remains unclear.

RpoS is present in WT and absent in Δ*rpoS*^{KAN} *E. coli* cells. To confirm the absence of RpoS in Δ*rpoS*^{KAN} cells, we next sought to conduct a Western blot analysis. To identify RpoS, protein lysates collected from WT and Δ*rpoS*^{KAN} cells in mid-exponential phase were subjected to SDS-PAGE and probed with anti-RpoS antibodies. As expected, a band corresponding to RpoS was present in the WT sample and absent in the Δ*rpoS*^{KAN} sample (Figure 5). Additionally, an RpoS Western blot analysis was conducted on WT *E. coli* cells treated with a gradient of sub-lethal concentrations of ampicillin (0.5, 1, 2, 4, and 8 µg/mL) and gentamicin (1, 2, 4 µg/mL). While we hypothesized that the increase in antibiotic concentration would result in a dose-dependent upregulation of RpoS, high background noise restricted quantification of the bands, thus rendering these results inconclusive (Figure S7). Consequently, we are only able to conclude that RpoS is in fact present in WT and absent in the Δ*rpoS*^{KAN} *E. coli* cells.

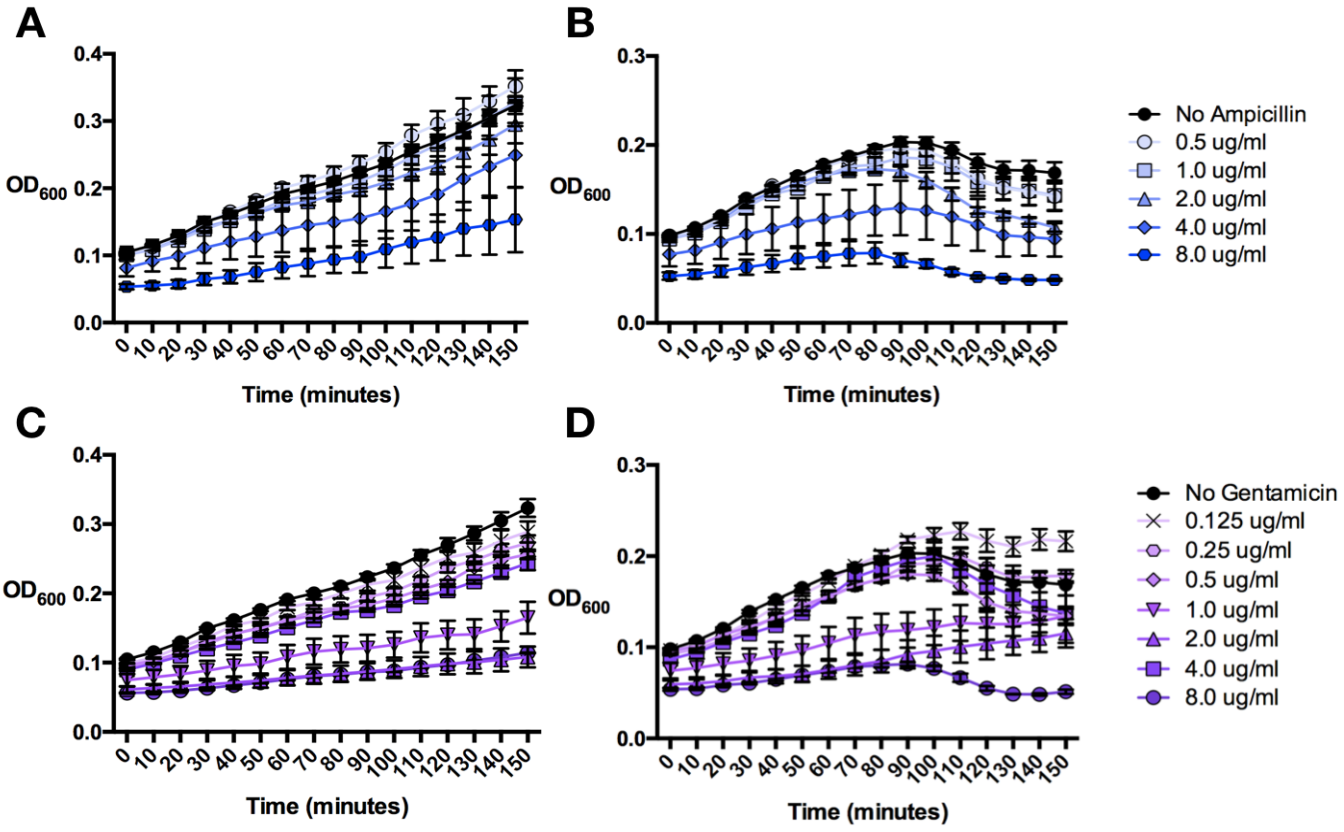

FIG. 4 Absence of RpoS does not affect rate of lysis by T7 phage in cells treated with a gradient of concentrations of ampicillin and gentamicin. (A) 96-well plate infectivity assay with *ΔrpoS*^{KAN} *E. coli* treated with a gradient of concentrations of ampicillin in the absence of added phage. Bars represent +/- SEM. n = 24 wells for No Ampicillin condition. n = 6 wells for all ampicillin-treated conditions. (B) Gradient of concentrations of ampicillin in the presence of added phage. Bars represent +/- SEM. n = 24 wells for No Ampicillin condition. n = 6 wells for all ampicillin-treated conditions. (C) Gradient of concentrations of gentamicin in the absence of added phage. Bars represent +/- SEM. n = 24 wells for No Gentamicin condition. n = 3 wells for 0.125 μg/mL, 0.25 μg/mL, 4 μg/mL and 8 μg/mL, and n = 6 wells for 0.5 μg/mL, 1 μg/mL, and 2 μg/mL. (D) Gradient of concentrations of gentamicin in the presence of added phage. Bars represent +/- SEM. n = 24 wells for No Gentamicin condition. n = 3 wells for 0.125 μg/mL, 0.25 μg/mL, 4 μg/mL and 8 μg/mL, and n = 6 wells for 0.5 μg/mL, 1 μg/mL, and 2 μg/mL.

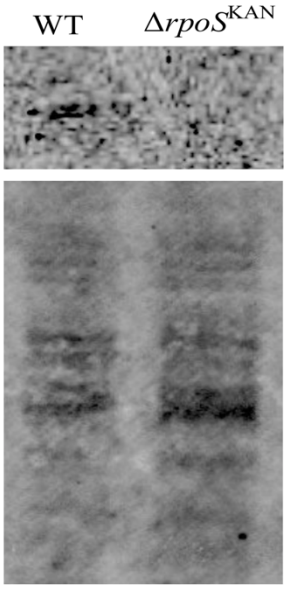

FIG. 5 RpoS is present in WT and absent in Δ*rpoS*^{KAN} *E. coli* cells. Western blot analysis of RpoS (top) and total protein loading control (bottom). WT and Δ*rpoS*^{KAN} cells were grown in LB. Membrane was blocked with 5% skim milk and probed with primary anti-RpoS and secondary anti-mouse antibodies. After treatment with detection reagents, membrane was visualized on the Bio-Rad ChemiDoc MP Imaging System.

DISCUSSION

Sub-lethal antibiotic concentration. In bacteria**,** sub-lethal antibiotic concentrations play an important role in gene expression and can often lead to antibiotic resistance (17, 18). Despite its importance, there is no agreed upon definition of "sub-lethal antibiotic concentration" (5, 17). As such, for our investigation, it was important to develop a working definition of sub-lethal in order to adequately assess potential antibiotic-induced cross-protection by RpoS.

Initially, in order to replicate the results presented by Li *et al.*, we chose to define sub-lethal from the results of our MIC assay (Table 2). However, upon testing these concentrations in the phage lysis assay conducted by Li *et al.*, these concentrations were shown to be too high, resulting in complete cell death (Figure S6). As a result, we revisited our definition of sub-lethal for subsequent experiments. If RpoS is indeed conferring cross-protection against T7 phage-induced lysis, the corresponding sub-lethal concentration of antibiotics needed to observe this phenotype should be the lowest concentration of antibiotic required to induce upregulation of RpoS compared to untreated cells. This led to the modification of the assay used by Li *et al.* (i.e. the gradient assay) in order to test varying concentrations of both ampicillin and gentamicin in a single phage lysis assay. The rationale for this assay was two-fold. First, identifying the lowest concentration of antibiotic needed to induce RpoS upregulation would allow us to minimize any non-specific effects on cell function that could potentially be induced by increased antibiotic stress. Second, this definition would allow us to quantify RpoS expression through Western blotting in order to more accurately compare the effects of antibiotic stress on a subcellular level and apply it to cross-protection.

While the Western blot analysis was able to confirm the absence of RpoS in Δ*rpoS*^{KAN} cells, it was unable to provide any conclusive evidence to identify the ideal sub-lethal concentration of either ampicillin or gentamicin to induce RpoS upregulation (Figures 5, S7). In addition, using the gradient assay, we were unable to observe delayed lysis in WT cells treated with sub-lethal levels of ampicillin and gentamicin (Figure 3).

An absence of delayed lysis. Contrary to the results presented by both Hardman *et al.* and Li *et al.*, we were unable to observe T7 phage-induced delayed lysis following exposure of cells to sub-lethal concentrations of ampicillin and gentamicin (6, 7). While our data conflicts with previous findings, we propose two explanations for this discrepancy.

First, deeper investigation into the data collected in these two aforementioned studies revealed questions regarding the significance of their findings. In Hardman *et al.*, while exponential phase *E. coli* UB1005 cells treated with sub-lethal gentamicin exhibited decreased T7 phage lysis, these results were not statistically significant due to a lack of sufficient replication (7). In Li *et al.*, several problems were identified. Most notably, the 96-well plate phage lysis assay conducted in their investigation was only performed once, leading

to a maximum number of three replicates for each tested condition. Furthermore, data suggesting delayed lysis with sub-lethal treatment of gentamicin was not presented with statistical analysis (6).

Second, similar to the results presented by Li *et al.*, a maximum delay of lysis by 10 minutes was observed for 0.25, 0.5, 2, 4, 8 µg/mL gentamicin-treated exponential phase WT cells (Figure 3D). While interesting, we remain skeptical of these results. Due to the design of our gradient phage lysis assay, OD_{600} readings were taken at intervals of 10 minutes. As such, it is possible that this observed delay of lysis by 10 minutes with select concentrations of gentamicin was due to the timing of readings, and not the result of cross-protection. Furthermore, in the case of the gradient assay conducted with WT cells, variability of the observed 10-minute delay between individual trials further supports our suspicion for the absence of delayed lysis. Additionally, in the case of the gradient assay conducted with $\Delta rpoS^{KAN}$ cells, the lack of sufficient replicates renders this data inconclusive (Figure 4D).

Ultimately, due to lack of conclusive evidence, we cannot provide support for delayed T7-induced lysis through the exposure of cells to sub-lethal concentrations of ampicillin and gentamicin. Furthermore, the lack of difference in lysis between WT and $\Delta rpoS^{KAN}$ cells suggests that RpoS may not be involved in the mediation of sub-lethal antibiotic-induced cross-protection during T7 phage infection.

An unclear role for RpoS in responding to antibiotic stress in exponential phase *E. coli*. In our investigation, pursuant to the results of a recent study, we examined the potential for antibiotic-induced cross-protection mediated by RpoS in exponential phase *E. coli* (6). Unbeknownst to us, however, several recent studies have cast doubt on the role of antibiotic stress in exponential phase cells, rendering this model far less definitive (19-21). In stationary phase, cells are faced with increased stress as nutrients become limiting and waste products begin to accumulate (22). Correspondingly, it has also been shown that RpoS may only be upregulated to sufficient levels as part of the bacterial general stress response in stationary phase cells (18, 19). Moreover, prior investigations have found numerous RpoS-dependent genes to be expressed in a growth phase-dependent manner, with dramatically higher

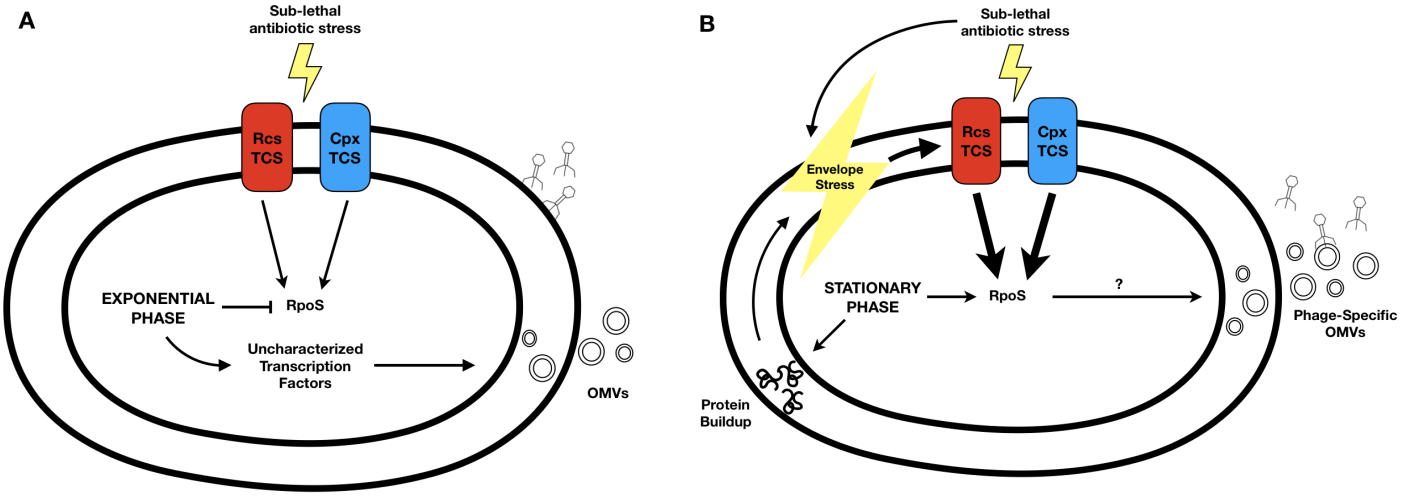

FIG. 6 Proposed model for interaction of *rpoS*-inducing two-component systems, antibiotic-mediated cell stress pathways, and T7 phage-mediated cell lysis. (A) Exponential phase cells limit *rpoS* expression in favour of other sigma factors. Upon exposure to sub-lethal antibiotic stress, the Rcs and Cpx TCSs are activated, resulting in the stabilization of the limited *rpoS* transcripts. Coincidentally, uncharacterized transcription factors mediate the production of factors needed to produce OMVs. These OMVs, though important to cell function, play no role in responding to T7 phage infection. (B) Stationary phase cells are exposed to conditions that activate *rpoS* expression. Upon exposure to sub-lethal antibiotic stress, the Rcs and Cpx TCSs are activated, resulting in the stabilization of the high level of *rpoS* transcripts. Simultaneously, protein buildups in the bacterial envelope caused by cell state and antibiotic stress lead to envelope stress. Envelope stress elicits a positive feedback loop on the Rcs and Cpx TCSs leading to increased RpoS production. Through an unknown mechanism, RpoS activates the transcription of factors needed to produce OMVs. These OMVs act as decoy membranes in order to delay T7 phage infection and cell lysis.

expression found in stationary phase cells regardless of media composition-induced stress (23). If true, these observations may suggest a stationary phase-specific response towards antibiotic stress not investigated in this study, and may explain the previously-discussed absence of any observed delayed lysis.

With regard to the role of RpoS in responding to antibiotics, a body of evidence has demonstrated its significance in responding to antibiotic stress. Consistent with our findings, it has been observed that the absence of *rpoS* can lead to greater antibiotic sensitivity, particularly in the case of gentamicin (19). In our investigation, lower concentrations of gentamicin were required for use in our gradient phage lysis assay with Δ*rpoS*^{KAN} compared to WT (Figures 3, 4). RpoS has also been observed to be upregulated in stationary cells in the presence of beta-lactam antibiotics, including ampicillin, via Western blot analysis (18). Despite our efforts to quantify the relative presence of RpoS in cells treated with sub-lethal concentrations of antibiotics via Western blot analysis, these results were inconclusive (Figures 5, S7). Although there was the option of optimizing the blots by stripping the reagents and reprobing, we were unable to redo this experiment due to time limitations. Finally, similar phenomena in response to aminoglycoside antibiotics have been observed in other bacterial species, including *Vibrio cholerae*, suggesting a conserved and well-established paradigm for the role of RpoS in responding to antibiotic stress (17). Taken together, these observations suggest that RpoS does indeed play a crucial role in the response to antibiotics, but perhaps not until stationary phase is reached.

The mechanisms of stress, sensing and responding to antibiotics, and T7 phage infection. Upstream of RpoS, two-component systems (TCSs) play an essential role in altering gene expression to mediate the response to antibiotics. In *E. coli*, two main TCSs have been shown to respond to antibiotic stress. The first, the regulator of the capsule synthesis (Rcs) pathway, mediates a signalling cascade to activate the transcription of a small RNA molecule known as *rprA* (1). *rprA* has been implicated in providing stability to the mRNA transcript of *rpoS* (1, 2). The second, the conjugative pilus expression (Cpx) TCS, is one of the most widely described envelope stress systems present in *E. coli* (24) and has been shown to play a role in *E. coli* biofilm formation, chemotaxis, resistance to antimicrobials, and, most recently, gentamicin resistance (24-26). Recent studies have also shown the existence of a direct interaction between the Cpx TCS and *rprA*, with a Cpx element acting as a positive regulator of *rprA*, and *rprA* acting as a negative feedback inhibitor directly back onto the TCS (27). In addition, the Cpx TCS has been implicated in the mediation of upregulation of another sigma factor induced during stress, RpoH, in response to gentamicin exposure in *E. coli* (26). While both of these TCSs play a role in *rprA* transcript activation to stabilize the transcript of *rpoS*, this stabilization may not be sufficient to upregulate RpoS in the cell. As RpoS is a stress factor associated with cells in stationary phase, genes and their encoded proteins regulated by the presence of RpoS are not present at high levels until stationary phase is reached (23). As such, even with the activation of the Rcs and Cpx TCSs as a result of antibiotic stress, RpoS may not be upregulated sufficiently until the cell has reached stationary phase. If true, this paradigm may explain the lack of any observable difference between exponential phase WT and *ΔrpoS*^{KAN} *E. coli* treated with ampicillin and gentamicin in our gradient phage lysis assays (Figures 3, 4).

Downstream of the Rcs and Cpx TCSs, a large proportion of Gram-negative bacteria have been shown to release small (20-300 nm) outer membrane vesicles (OMVs) as part of the general stress response (28). Consisting of a small spherical lipid bilayer and internal aqueous materials, OMVs are a diverse and versatile tool used by Gram-negative bacteria like *E. coli* for defense and protection during periods of stress. (29). Notably, OMVs can also be modified in terms of their amount and composition depending on internal and external factors, including the growth phase of the cell and antibiotic stress (29, 30). Furthermore, both the Rcs and Cpx TCSs have been shown to play a role in the production of OMVs via their respective signalling pathways (30, 31). This often occurs in response to the buildup of proteins in the bacterial envelope as a result of antibiotics or similar stress, in an event known as ‘envelope stress’ (32, 33). Similar to the previously discussed stationary phase-specific nature of RpoS, increased envelope stress due to the accumulation of misfolded proteins may also be a phenomenon unique to stationary-phase cells (29). Finally**,** OMVs have also been

implicated in mediating resistance to phage infection in stationary cells, whereby OMVs covered with LPS—the primary receptor of both T7 and T4 phage—act as decoys, preventing phage from binding to the cell envelope to insert its DNA (34, 35). Though no studies have been done to directly link T7 phage resistance to OMVs, studies investigating T4 phage have found infection to be significantly reduced in the presence of OMVs (36). As such, the key to observing the delayed lysis may hinge upon activity of OMVs, not RpoS.

Given what is known about RpoS, antibiotic-mediated cell stress, T7 phage-induced lysis, and the results presented in this investigation, we propose the following model (Figure 6). In exponential phase, the transcription of *rpoS* is limited in favour of other sigma factors (Figure 6A). In the case of sub-lethal antibiotic stress, both the Rcs and Cpx TCSs are activated, resulting in a *rprA*-dependent stabilization of the comparatively small number of *rpoS* transcripts present in the cell. Concurrently, as-of-yet uncharacterized transcription factors transcribe genes needed for the production of OMVs. Though critical to cell function, these OMVs do not display the necessary LPS and other membrane-bound factors to prevent phage binding to the cell.

In stationary phase, factors associated with this cell state, such as waste and limited nutrient availability, activate the transcription of *rpoS* (Figure 6B). In the case of sub-lethal antibiotic stress, both the Rcs and Cpx TCSs are activated, resulting in an *rprA*-dependent stabilization of the high level of *rpoS* transcripts present in the cell. Concurrently, protein buildup in the bacterial envelope and subsequent envelope stress induced by both stationary phase factors and sub-lethal antibiotics induces a positive feedback loop onto RpoS production via the Rcs and Cpx TCSs. While more work is needed to elucidate the role, if any, of RpoS in the production of OMVs, we further propose that RpoS activates the transcription of factors needed to produce OMVs tagged with sufficient LPS and other membrane-bound factors to prevent phage binding to the cell, resulting in delayed cell lysis.

In conclusion, we modify an existing phage lysis assay to examine the effect of a gradient of sub-lethal antibiotic concentrations on cross-protection against T7 phage-induced lysis. We show that the previously-reported evidence of delayed T7 phage-induced lysis may not occur in ampicillin and gentamicin-treated exponential phase cells. In comparison to WT, we show that the loss of RpoS does not result in greater susceptibility to T7 phage-induced lysis, suggesting that RpoS may not be necessary for the mediation of cross-protection in exponential phase cells. As a result, we propose a complex model of cross-protection, whereby sub-lethal antibiotics, cell growth phase, stress, and OMVs all play an unknown role in a larger system of bacterial response to stress. Continued investigation into all aspects of this intricate system is required in order to further elucidate how bacteria respond to stress.

Limitations Due to using a micropipette instead of a multichannel pipette, there was up to a 10-minute difference between the point at which phage was added to the first well and the point at which it was added to the last well. To address this lack of uniformity in exposure time, we present two possible solutions. First, the order in which phage is added to the wells could be randomized from trial to trial. Second, a reliable multichannel pipette could be used to decrease the time it takes to dispense phage into the wells. In addition, to address any issues related to the repeated removal of the plate from the incubator during the phage assays, the OD_{600} could be measured by using a plate reader with a built-in incubation feature.

Future Directions In our investigation, we were unable to demonstrate the presence of delayed T7 phage-induced lysis mediated through RpoS-dependent cross-protection. Despite our lack of evidence in support of this phenomenon, we believe this area of study to still be of great importance. In particular, a better understanding of the general stress response in bacteria will give better insight into the design of treatments for bacterial infection. For future groups interested in the study of RpoS, it would first be important to attempt a similar gradient assay as performed here with both WT and Δ*rpoS*^{KAN} cells in stationary phase. In addition, though far more elaborate, it would be of interest to examine the potential role for RpoS in the regulation of factors that produce OMVs as part of the stationary phase stress response. Though we predicted that our investigation focused on RpoS would yield the most conclusive results, in reality, consultation with the existing literature revealed a far more complex model system that cannot be elucidated through the study of RpoS alone.

Beyond RpoS, we strongly encourage future groups to examine the mechanisms both up and downstream of this sigma factor in response to sub-lethal antibiotic concentrations and T7 phage. As previously mentioned, special consideration must be given to the growth phase of investigated cells, as previous studies have demonstrated profound differences in the general stress response between bacteria in exponential phase and those in stationary phase, particularly with regard to RpoS expression (18, 19).

Looking upstream of RpoS, though both the Rcs and Cpx TCSs have been suggested to play a role in responding to antibiotics, more definitive data is needed to see which, if any, varieties of antibiotics these two TCSs respond to (1, 26). In addition, more research is needed to elucidate the role of these TCSs in responding to envelope stress in stationary phase cells. Downstream of RpoS, there remains much to be explored regarding OMVs. Pertaining to cell growth phase, quantification of the relative levels and bilayer contents of these OMVs would provide valuable insight into our previously proposed model (Figure 6). With regard to delayed lysis and OMVs, it would be invaluable to ascertain exactly what factors and their relative quantities elicit the aforementioned reduced infectivity towards T4 phage (36). Following this, using OMVs to confirm reduced infectivity of T4 phage and attempting similar assays with T7 phage will provide further insight into this complex secretory system.

ACKNOWLEDGEMENTS

We would like to thank Dr. David Oliver, Ashley Arnold, and Gyles Ifill for sharing their expertise and providing unparalleled support over the course of this project. We would also like to thank the media room staff, Dr. John Nomellini, and Meysam Abbasi from Bio-Rad Laboratories. Finally, we would like to extend our gratitude to the Department of Microbiology and Immunology at the University of British Columbia for providing the funding to make this project possible.

REFERENCES

1. Wang J, Woo M, Yan C. 2017. Role of the Rcs Phosphorelay in Intrinsic Resistance to Penicillin, Phosphomycin, and Cefsulodin in *Escherichia coli* K12. J Exp Microbiol Immunol, In Press. 21:60-66.
2. Majdalani N, Hernandez D, Gottesman S. 2002. Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. Mol Microbiol 46:813–826.
3. Chung HJ, Bang W, Drake MA. 2006. Stress Response of *Escherichia coli*. Compr Rev Food Sci Food Saf 5:52-64. doi:10.1111/j.1541-4337.2006.00002.x
4. Pelzek AJ, Schuch R, Schmitz JE, Fischetti VA. 2013. Isolation, Culture, and Characterization of Bacteriophages. Curr Protoc Essential Lab Tech 7: 4.4.1-4.4.33
5. You L, Suthers PF, Yin J. 2002. Effects of *Escherichia coli* Physiology on Growth of Phage T7 *in vivo* and *in silico*. J Bacteriol 184:1888–1894.
6. Li H, Wang W, Yang Y, Lam M. 2017. Sub-lethal treatment of *Escherichia coli* strain B23 with either β-lactam or Aminoglycoside Antibiotics May Delay T7 Bacteriophage-mediated Cell Lysis. J Exp Microbiol Immunol, In Press.
7. Hardman B, Hunt DJ, Mojaab D, Naor A. 2017. Sub-Lethal Gentamicin Treatment of *Escherichia coli* UB1005 Induces the Release of Soluble Factors that Reduce Susceptibility to T7 Bacteriophage Infection. J Exp Microbiol Immunol, In Press. 21:128-133.
8. Fettig N, Ryken A, Mar N, Sunario A. 2017. Growth phase of host *Escherichia coli* MG1655 influences T4 and T7 bacteriophage replication patterns during coinfection. J Exp Microbiol Immunol, In Press.
9. Francis F, Miller R, Nagra M. 2017. Phage overlay assay for quantification of infectious bacteriophage particles. J Exp Microbiol and Immunol Methods 1:18-21.
10. Thermo Fisher Scientific. 2015. User Guide: Platinum™ *Taq* DNA Polymerase.
11. Hay M, Li YM, Ma Y. 2017. Deletion of AcrS Results in Increased Expression of *acrE* and Confers an Increase in Kanamycin Resistance in *Escherichia coli* BW25113. J Exp Microbiol Immunol Pearls 3:63-69.
12. Bio Basic. 2015. EZ-10 Spin Column Handbook.
13. Barrick J. 2010. Making electrocompetent *E. coli* cells (large batch).
14. Barrick J. 2017. Transforming *E. coli* cells by electroporation.
15. Barrick J. 2017. FLP Recombination in *E. coli*.
16. Bio-Rad Laboratories. 2010. Trans-Blot® Turbo™ Blotting System: Instruction Manual.
17. Baharoglu Z, Krin E, Mazel D. 2013. RpoS plays a central role in the SOS induction by sub-lethal aminoglycoside concentrations in *Vibrio cholerae*. PLoS Genet 9:e1003421.

18. Gutierrez A, Laureti L, Crussard S, Abida H, Rodriguez-Rojas A, Blázquez J, Baharoglu Z, Mazel D, Darfeuille F, Vogel J. 2013. β-Lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. Nat Commun 4:1610.
19. Wang J-H, Singh R, Benoit M, Keyhan M, Sylvester M, Hsieh M, Thathireddy A, Hsieh Y-J, Matin AC. 2014. Sigma S-Dependent Antioxidant Defense Protects Stationary-Phase *Escherichia coli* against the Bactericidal Antibiotic Gentamicin. Antimicrob Agents and Chemother 58:5964–5975.
20. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. 2013. Killing by Bactericidal Antibiotics Does Not Depend on Reactive Oxygen Species. Science 339:1213–1216.
21. Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CT, Lobritz MA, Braff D, Schwarz EG. 2014. Antibiotics induce redox-related physiological alterations as part of their lethality. Proc Natl Acad Sci USA 111:E2109.
22. Boor KJ. 2006. Bacterial stress responses: what doesn't kill them can make them stronger. PLoS Biol 4:e23.
23. Vijayakumar SRV, Kirchhof MG, Patten CL, Schellhorn HE. 2004. RpoS-Regulated Genes of *Escherichia coli* Identified by Random *lacZ* Fusion Mutagenesis. J Bacteriol 186:8499–8507.
24. Surmann K, Ćudić E, Hammer E, Hunke S. 2016. Molecular and proteome analyses highlight the importance of the Cpx envelope stress system for acid stress and cell wall stability in *Escherichia coli*. Microbiologyopen 5:582–596.
25. Weatherspoon-Griffin N, Yang D, Kong W, Hua Z, Shi Y. 2014. The CpxR/CpxA two-component regulatory system up-regulates the multidrug resistance cascade to facilitate *Escherichia coli* resistance to a model antimicrobial peptide. J Biol Chem 289:32571–32582.
26. Ćudić E, Surmann K, Panasia G, Hammer E, Hunke S. 2017. The role of the two-component systems Cpx and Arc in protein alterations upon gentamicin treatment in *Escherichia coli.* BMC Microbiol 17:197
27. Vogt SL, Evans AD, Guest RL, Raivio TL. 2014. The Cpx Envelope Stress Response Regulates and Is Regulated by Small Noncoding RNAs. J Bacteriol 196:4229–4238.
28. Kulkarni HM, Nagaraj R, Jagannadham MV. 2015. Protective role of *E. coli* outer membrane vesicles against antibiotics. Microbiol Res 181:1–7.
29. Schwechheimer C, Kuehn MJ. 2013. Synthetic Effect between Envelope Stress and Lack of Outer Membrane Vesicle Production in *Escherichia coli.* J Bacteriol 195:4161–4173.
30. Mcbroom AJ, Kuehn MJ. 2006. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. Mol Microbiol 63:545–558.
31. Mcmahon KJ, Castelli ME, Vescovi EG, Feldman MF. 2012. Biogenesis of Outer Membrane Vesicles in *Serratia marcescens* Is Thermoregulated and Can Be Induced by Activation of the Rcs Phosphorelay System. J Bacteriol 194:3241–3249.
32. Laubacher ME, Ades SE. 2008. The Rcs Phosphorelay Is a Cell Envelope Stress Response Activated by Peptidoglycan Stress and Contributes to Intrinsic Antibiotic Resistance. J Bacteriol 190:2065–2074.
33. Mahoney TF, Silhavy TJ. 2013. The Cpx Stress Response Confers Resistance to Some, but Not All, Bactericidal Antibiotics. J Bacteriol 195:1869–1874.
34. Washizaki A, Yonesaki T, Otsuka Y. 2016. Characterization of the interactions between *Escherichia coli* receptors, LPS and OmpC, and bacteriophage T4 long tail fibers. Microbiologyopen 5:1003–1015.
35. Kulp A, Kuehn MJ. 2010. Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles. Annu Rev of Microbiol 64:163–184.
36. Manning AJ, Kuehn MJ. 2011. Contribution of bacterial outer membrane vesicles to innate bacterial defense. BMC Microbiol 11:258.